Cell Stem Cell
Minireview

MicroRNA Gene Regulatory Pathways in the Establishment and Maintenance of ESC Identity

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Understanding the molecular foundations of embryonic stem cell (ESC) self-renewal and pluripotency will facilitate therapeutic exploitation of these remarkable cells. Here we discuss the emerging roles of microRNAs in the establishment and maintenance of ESC identity and summarize our current understanding of the mechanisms controlling microRNA expression and function in ESCs.

Embryonic stem cells (ESCs) derived from the inner cell mass (ICM) of mammalian blastocyst-stage embryos are characterized by two properties: the ability to differentiate into any type of specialized cell, known as pluripotency, and their unlimited capacity for self-renewal. ESCs can be isolated and cultured in vitro without losing their ability to contribute to all cell types, and thus hold big promise for future studies of tissue formation and drug development as well as cell replacement therapy. Furthermore, the capacity to artificially reprogram differentiated cells back into a stem cell-like state provides great potential for regenerative medicine. Pluripotency and self-renewal are intricate biological processes that are coordinately regulated by a complex set of factors. ESC self-renewal requires that the unique molecular program of the pluripotent state be maintained. In contrast, to differentiate into various cell lineages, ESCs must shift to alternative molecular programs that inhibit self-renewal and promote the differentiated state. How cells switch between pluripotency and differentiation is still incompletely understood. However, several "stemness" factors required to ensure appropriate ESC behavior have been identified. A core network of transcription factors and RNA-binding proteins, including Oct4, Sox2, Nanog, Klf4, c-Myc, Tcf3, and Lin28, cooperate in intricate regulatory circuits to ensure appropriate ESC behavior (Marson et al., 2008). Some of these factors share a substantial fraction of their target genes and participate in autologous feedback loops to control one another's transcription. Notably, several of these key transcription factors directly regulate microRNA (miRNA) expression in ESCs. By occupying miRNA gene promoter regions, these factors not only activate expression of ESC miRNAs but also play a role in silencing a subset of miRNAs that are expressed in differentiated cell types. When artificially expressed in differentiated cells, a subset of these regulatory factors can reprogram cells back to a stem cell-like state, called induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Yu et al., 2007). Numerous lines of evidence now implicate miRNAs as central players in ESC biology (Gangaraju and Lin, 2009). In this article, we discuss the emerging roles of miRNAs in the self-renewal and differentiation of ESCs and summarize our current understanding of the complex mechanisms controlling miRNA expression and function in embryonic stem cells.

miRNA Biogenesis and Function

miRNAs are now recognized as an abundant class of genome-encoded, small RNAs that repress gene expression at the post-transcriptional level via base pairing to complementary sites located in target messenger RNAs (mRNAs) (Winter et al., 2009). miRNAs are evolutionarily conserved and have been implicated in the regulation of a variety of diverse biological processes. Dysregulated miRNA expression is associated with several diseases, most notably cancer (Esquela-Kerscher and Slack, 2006). The biogenesis of most of the several hundred known miRNAs begins with the transcription of long, capped, and polyadenylated primary transcripts (pri-miRNA) by RNA polymerase II. Hairpin-shaped pri-miRNAs are cleaved in the nucleus by the Microprocessor complex, comprised of the RNase III enzyme Drosha and the double-stranded RNA binding domain (dsRBD) protein DGCR8. The Microprocessor specifically cleaves pri-miRNAs at the base of the stem loop to produce ~60–70 nt long precursors (pre-miRNA) that are subsequently transported by Exportin-5 to the cytoplasm where they are recognized by a heterotrimeric complex composed of the RNase Dicer, the double-stranded RNA-binding protein TRBP, and Argonaute (Ago) proteins. Dicer further cleaves pre-miRNAs ~22 nt from the Drosha cleavage site, thereby generating a mature miRNA duplex. This complex identifies the guide strand of the ~22 nt RNA duplex and separates the two RNA strands. The guide strand of the miRNA remains associated with the Ago protein in the miRNA-induced silencing complex (miRISC), which in turn recognizes target mRNAs based on complementarity between the miRNA and the mRNA target (Winter et al., 2009). Nucleotides 2–7 (from the 5′-end) of the mature miRNA, also called the "seed" motif, form the critical region for target mRNA recognition that hybridizes nearly perfectly with the target to nucleate the miRNA-mRNA interaction. In this way a particular miRNA sequence is thought to guide the regulation of several hundred different mRNAs. Additionally, an individual mRNA may be simultaneously targeted by multiple different miRNAs. Thus, miRNA-mediated gene regulation represents an extensive mechanism for modulating gene expression. Although still controversial, mRNA-mediated posttranscriptional gene silencing may involve translational repression, degradation,

and/or deadenylation of target mRNAs, as well as cotranslational protein degradation.

Global Loss of miRNAs Results in Proliferation Defects and a Failure to Silence the ESC Program

The overall function of the miRNA pathway in mouse ESCs has been evaluated by analyzing the phenotypes caused by genetic ablation of essential components of the miRNA biogenesis machinery. *Dicer1*-knockout mice die at early stages of development and Dicer-deficient mouse ESCs are defective in differentiation (reviewed by Gangaraju and Lin, 2009). Specifically, loss of Dicer in ESCs leads to an acute loss of proliferative potential that can eventually be rescued by the accumulation of compensatory factors, either mutations or stable changes in gene expression. Interestingly, these cells display an altered cell cycle profile, with a slight increase of cells in the G1 and G0 (nondividing) phases of the cell cycle. Because Dicer is required for the maturation of other types of small RNAs besides miRNAs, a more accurate interpretation of the role of miRNAs in ESCs came from *Dgcr8*-knockout studies. Mice deficient in DGCR8 also arrest in early development and, although less severe than *Dicer1*-deficient cells, *Dgcr8*-knockout ESCs also exhibit a proliferation defect and cannot efficiently silence the ESC program. Even under stringent differentiation conditions, DGCR8-deficient ESCs do not fully downregulate pluripotency markers and retain the ability to produce ESC colonies (Gangaraju and Lin, 2009).

Similar to miRNA-deficient mouse ESCs, *Dicer*- and *Drosha*-knockdown human ESCs, which reportedly appear morphologically similar to their wild-type counterparts, are unable to downregulate stem cell-specific markers and display defects in self-renewal. Specifically, *Dicer*- and *Drosha*-knockdown human ESCs are characterized by G1-S and G2-M transition delays (Qi et al., 2009). Altogether, these findings indicate that miRNAs are required for proper function of both proliferation and differentiation pathways in mouse and human ESCs.

ESCs Are Characterized by a Defined miRNA Signature

Small RNA profiling in different mammalian tissues and cell types has revealed a plethora of miRNAs, with more than 500 identified so far, some of which are cell type specific and others of which display a more widespread expression profile. Both mouse and human ESCs express only a very limited repertoire of miRNAs whose levels decrease as the stem cells differentiate (Gangaraju and Lin, 2009). Indeed, the ESC-specific miR-290 family (which includes miR-290, miR-291a, miR-291b, miR-292, miR-293, miR-294, miR-295) and miR-302 cluster (miR-302a, miR-302b, miR-302c, miR-302d, miR-367) in mice, and miR-371 family (miR-371, miR-372, miR-373) that is homologous to the mouse miR-290 family, and miR-302 family in humans, represent the majority of the total miRNA molecules expressed in undifferentiated ESCs (Marson et al., 2008). Interestingly, many of the ESC-specific miRNAs are cotranscribed as polycistronic transcripts, suggesting common upstream regulation and coordinate expression patterns. Indeed, the transcription of these miRNAs seems to be directly regulated by a core set of pluripotency transcription factors (Marson et al., 2008). Significantly, members of this set of ESC miRNAs possess the same or similar seed motif, suggesting common sets of target mRNAs (Gangaraju and Lin, 2009). Furthermore, ESC-expressed miRNAs are not present or exist only at very low levels in somatic cells. Conversely, a number of miRNAs that appear to be widely expressed in most differentiated cell types, notably the let-7 family, are present at low levels in ESCs (Viswanathan et al., 2008).

ESC-Specific miRNAs Regulate the Cell Cycle

ESCs are highly proliferative and exhibit an expedited cell cycle, which is critical for fast growth during early mammalian embryogenesis. The average cell cycle length of mouse ESCs is around 12 hr as opposed to ~24 hr for somatic cells. The cell cycle of a dividing cell is composed of four consecutive phases: G1, S, G2, and M. ESCs exhibit a unique cell cycle structure in which a shortened G1 phase is maintained. This pattern is accomplished by an unrestricted G1 to S phase transition (G1-S). The G1 restriction point normally requires the sequential activation of the Cdk4/6 and the Cdk2 kinases by the D and E type cyclins, respectively. In mouse ESCs, the cyclin D-Cdk4/6 complex is absent, and progression though the G1-S transition is primarily controlled by the constitutive activity of the cyclin E-Cdk2 complex. Upstream inhibitors including Cdkn1a, Cdkn1b, Rb1, Rbl1, Rbl2, and Lats2 modulate the activity of the Cdk-Cyclin complexes (Wang et al., 2008).

The proliferation defect of *Dicer1* and *Dgcr8* mutant ESCs suggests that miRNAs are normally required to promote the G1-S transition, perhaps by repressing proteins that directly or indirectly maintain ESC self-renewal. As predicted from a study of *Dicer1*-knockout ESCs, where it was shown that reintroduction of miR-290 cluster miRNAs can partially rescue the proliferation defects of miRNA-deficient ESCs (Sinkkonen et al., 2008), Blelloch and colleagues identified a set of miRNAs that partially rescue the proliferation and cell cycle defects of *Dgcr8*-deficient mouse ESCs (Wang et al., 2008). These miRNAs include members of the highly expressed miR-290 and miR-302 clusters that have been dubbed embryonic stem cell-cell cycle regulating (ESCC) miRNAs. ESCC miRNAs share a similar seed sequence, suggesting that common sets of genes are regulated by these miRNAs (Wang et al., 2008). In support of this model, cyclin E-Cdk2 upstream inhibitors including Cdkn1a, Rb1, Rbl1, Rbl2, and Lats2 are upregulated in *Dgcr8*-knockout cells and are predicted targets of ESCC miRNAs. Moreover, a direct miRNA-mediated repression has been shown for Cdkn1a, Rbl2, and Lats2 (Sinkkonen et al., 2008; Wang et al., 2008). Hence, ESCC miRNAs directly repress key regulators of the cell cycle to ensure a fast G1-S transition. The miRNA redundancy highlights the importance of speeding up the G1-S transition in early embryonic cells.

Interestingly, the promoters of ESCC miRNA clusters are bound by core ESC transcription factors. Oct4, Sox2, Nanog, c-Myc, and Tcf3 directly bind and upregulate the expression of ESCC cell miRNAs (Marson et al., 2008). In turn, the ESCC miRNAs were shown to maintain the ESC program by inhibiting the epigenetic silencing of pluripotency factors. Specifically, the mir-290 cluster was shown to indirectly repress the expression of de novo DNA methyl-transferases by silencing the transcriptional repressor Rbl2 (reviewed by Gangaraju and Lin, 2009; Sinkkonen et al., 2008). These findings highlight the intricacy of regulatory circuits that ensure appropriate ESC behavior.

Analogous to mouse ESCs, their human counterparts are also characterized by an expedited cell cycle progression via

a shortened G1 phase. Although many of the cell cycle players are differentially expressed between human and mouse ESCs, the role of miRNAs in cell cycle progression seems to be conserved. For instance, miR-92a has been identified as a regulator of the G1-S transition in human ESCs by repressing the Cdkn1c checkpoint gene (Sengupta et al., 2009). Work by the Ruohola-Baker group demonstrated that two miRNAs, miR-195 and miR-372, could partially reverse the cell cycle delay in *Dicer*-knockdown human ESC lines. Whereas miR-372 was shown to regulate the G1-S checkpoint inhibitor Cdkn1a, miR-195 was shown to regulate the G2-M checkpoint inhibitory kinase WEE1, one of the three kinases that negatively regulate the G2 cyclin B-Cdk complex in mammalian cells (Qi et al., 2009).

The miRNA Pathway Regulates ESC Differentiation

During mammalian development, ESCs must shift to alternative molecular programs that inhibit self-renewal and orchestrate differentiation into highly specialized cell types. miRNA-deficient ESCs have defects in both normal proliferation as well as ESC differentiation (Gangaraju and Lin, 2009). Interestingly, ESCC miRNAs do not rescue the differentiation defects of *Dicer1*- or *Dgcr8*-knockout cells, suggesting that different miRNAs underlie the two phenotypes (Sinkkonen et al., 2008; Wang et al., 2008). The let-7 family of miRNAs is broadly expressed across differentiated tissues and is tightly regulated during ESC differentiation (Viswanathan et al., 2008). Therefore, it has been proposed that let-7 is a prodifferentiation factor with "anti-stemness" properties. Indeed, it has recently been demonstrated that when introduced into *Dgcr8*-knockout cells, let-7 rescues the capacity to silence the self-renewal program under differentiation conditions (Melton et al., 2010). It was found that let-7 induces the differentiation of mouse ESCs by directly targeting the expression of several stemness factors, including c-Myc, Sall4, and Lin28, all of which have let-7 binding sites in their 3′UTR (Melton et al., 2010). Remarkably, wild-type ESCs were found to be resistant to let-7-induced suppression of self-renewal. Moreover, cointroduction of the ESCC miRNAs of the miR-290 family inhibited the capacity of let-7 to silence self-renewal in *Dgcr8*-knockout cells. Though the mechanism underlying this phenomenon remains unaddressed, it is assumed that the ESCC miRNAs repress expression of an unidentified key factor (or factors) that would otherwise repress expression of certain stemness factors including c-Myc and Lin28 and that persistent expression of the stemness factors somehow makes these cells resistant to the differentiation-promoting action of let-7. It is likely that c-Myc plays an important role in this miRNA-mediated cell fate switch because, unlike wild-type ESCs, *c-Myc*$^{-/-}$ ESCs respond to introduction of let-7 miRNA and (at least partially) downregulate expression of pluripotency genes (Melton et al., 2010). Furthermore, expression of Lin28 is regulated directly by transcriptional activation by c-Myc. These studies indicate that the let-7 miRNA family opposes the function of ESCC miRNAs by repressing expression of many of the same downstream target genes that are indirectly activated by the ESCC miRNAs. These findings form the basis of a model proposing that different types of miRNA have opposing effects on the fate of ESCs: one type (ESCC miRNAs) promotes self-renewal and antagonizes differentiation and the other (let-7 family) promotes differentiation. The role of miRNAs in the regulation of cell fate decisions is discussed further in an accompanying minireview (Ivey and Srivastava, 2010).

miRNAs Control the Expression of Pluripotency Factors

Other miRNAs besides let-7 contribute to the restricted expression of pluripotency factors during stem cell differentiation. A study in murine ESCs has uncovered evidence for the miR-134-, miR-296-, and miR-470-mediated regulation of the pluripotency factors Nanog, Oct4, and Sox2 (Tay et al., 2008). In addition, work by Wellner and colleagues has shown that miR-200c, miR-203, and miR-183 cooperate to repress the pluripotency factors Sox2 and Klf4 in mouse ESCs (Wellner et al., 2009). Introduction of these miRNAs into undifferentiated ESCs was shown to upregulate differentiation markers and reduce the ability to self-renew. Likewise, miR-145 reportedly represses the pluripotency machinery of human ESCs (Xu et al., 2009). Enforced expression of miR-145 in human ESCs was shown to block self-renewal and induce expression of differentiation markers. Conversely, inhibition of miR-145 by the introduction of antisense oligonucleotides was shown to increase the self-renewal capabilities of human ESCs. In a double negative feedback circuit, OCT4 transcriptionally represses miR-145 expression. Oct4/Sox2/Nanog/Tcf3 similarly occupy a set of several different miRNA genes that are transcriptionally silenced in ESCs and are selectively activated in particular differentiated cell types. The promoter regions of these miRNA genes are co-occupied by repressive Polycomb group proteins and are associated with the inactive histone modification, H3K27me3, that is catalyzed by Polycomb group proteins (Marson et al., 2008). The observation that multiple miRNAs target the ESC transcriptional network to promote cell differentiation could reflect a functional reinforcement to commit to the differentiated state. Alternatively, it could reflect a commitment driven by each miRNA to differentiate along different developmental pathways or during different time periods.

The Role of miRNAs in Cell Reprogramming

Reprogramming to iPSCs is an inefficient process typically achieved by the introduction of the key pluripotency transcription factors Oct4 and Sox2 into somatic cells. Other stemness factors such as Klf4, c-Myc, Lin28, and Nanog, although not essential, can markedly increase the efficiency of reprogramming (Takahashi and Yamanaka, 2006; Yu et al., 2007). Recently, miRNAs were found to play a role in reprogramming fibroblasts to iPSCs (Judson et al., 2009). Mouse ESCC miRNAs were found to enhance the efficiency of Klf4-, Oct4-, and Sox2-induced reprogramming to an extent similar to c-Myc. Although the mechanism for how ESCC miRNAs can replace c-Myc in reprogramming is not entirely clear, it has been shown that ESCC miRNAs are directly upregulated by c-Myc (in combination with other core transcription factors), suggesting that they are downstream effectors of c-Myc-promoted pluripotency (Marson et al., 2008). Accordingly, these miRNAs did not further enhance reprogramming in the presence of c-Myc. However, unlike exogenous c-Myc, these miRNAs induced a homogeneous population of reprogrammed colonies, suggesting overlapping and independent functions of c-Myc and the miRNAs during induced pluripotency (Judson et al., 2009). It seems likely that the main contribution of ESCC miRNAs in cell reprogramming is to promote cell cycle progression. Conversely, suppression of the

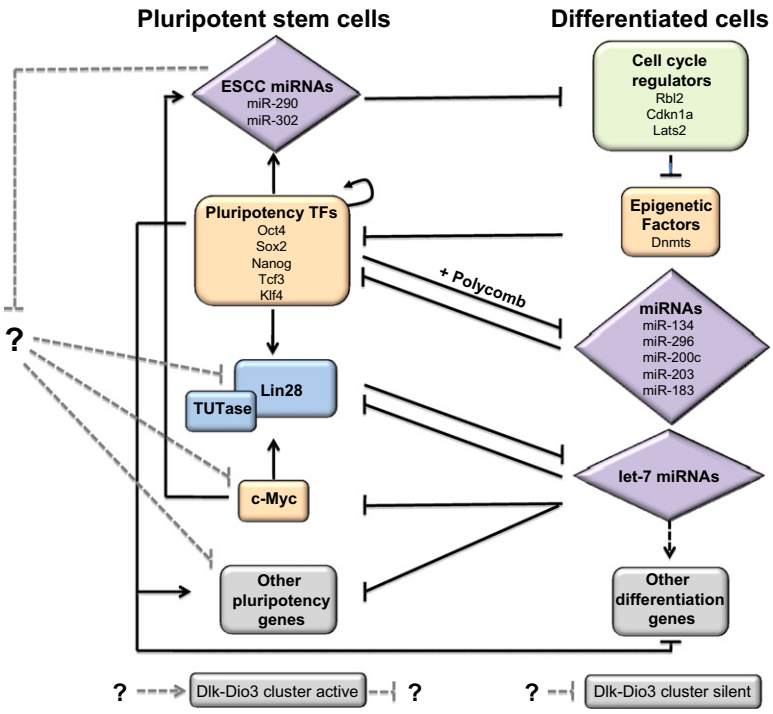

Figure 1. Regulatory Circuits Controlling ESC Identity
miRNAs are an integral part of the gene networks that regulate self-renewal and pluripotency of mouse embryonic stem cells (ESCs). Rectangles, proteins; diamonds, miRNAs; arrows, activation; blunted arrows, repression.

let-7 family of miRNAs via let-7 antisense inhibitors was found to modestly enhance reprogramming of mouse fibroblasts to iPSCs, highlighting the potential importance of this miRNA family in maintaining the differentiated state (Melton et al., 2010). These studies suggest that manipulation of miRNA levels might be a plausible route for generating iPSCs.

Regulation of let-7 miRNA Biogenesis by Lin28

As previously discussed, let-7 miRNA is an important regulator of stem cell differentiation. It was recently identified that ESCs possess an elaborate control mechanism to restrict expression of let-7. Earlier reports indicated that the expression of let-7 miRNA is regulated posttranscriptionally during mouse embryonic development and during differentiation of mouse ESCs. Indeed, mature let-7 family miRNAs (let-7-a1, -a2, -a3, -b, -c, -d, -e, -f1, -f2, -g, -i, and miR-98) are undetectable in ESCs and strongly accumulate 10 days after the onset of differentiation, whereas the corresponding pri-let-7 transcripts (from which the mature let-7 miRNAs derive) are readily detectable in both ESCs and differentiated cell types. The developmentally regulated RNA-binding protein Lin28 was recently identified as an inhibitor of let-7 expression (Viswanathan et al., 2008). Lin28 and Lin28B are orthologs of *C. elegans* LIN-28, a gene required for normal cell fate transitions and embryonic developmental timing. These proteins contain an N-terminal cold shock domain (CSD) and two Cys-Cys-His-Cys (CCHC)-type zinc fingers in the C terminus of the protein. Indeed, both domains seem to be required for RNA binding and Lin28 function. Lin28 and Lin28B selectively bind to let-7 precursor RNAs and inhibit the expression of the let-7 family (reviewed by Winter et al., 2009).

How does Lin28 inhibit the maturation of let-7 miRNA? Though it was found that Lin28-binding to let-7 precursor RNAs can inhibit the in vitro cleavage activity of both the Microprocessor and Dicer, it seems likely that the mechanism in vivo is more complex (Winter et al., 2009). Recently, Narry Kim's group and our laboratory independently identified a 3′ terminal uridylyl transferase (TUTase) that functions together with Lin28 to catalyze the addition of a short stretch of uridines to the 3′-end of the Lin28-bound pre-let-7 RNAs. This TUTase, called Zcchc11 (also known as TUT4), was identified as the enzyme responsible for the posttranscriptional modification of pre-let-7 RNA (Hagan et al., 2009; Heo et al., 2009). Zcchc11/TUT4 is a cytoplasmic TUTase that contains two poly(A) polymerase domains, a nucleotidyltransferase domain and several CCHC-type zinc finger domains. It belongs to a family of noncanonical poly(A) polymerases. Lin28 recruits Zcchc11/TUT4 to pre-let-7, and this oligouridylation activity is required for the blockade of let-7 maturation in ESCs. It is currently not clear how the Zcchc11/TUT4-mediated oligouridylation of pre-let-7 inhibits let-7 biogenesis, but the current model implicates an unidentified ribonuclease that recognizes oligo(U) tails as a signal inducing pre-let-7 degradation, similar to the mechanism reported for histone H1 mRNA turnover (Winter et al., 2009).

What is the role of Lin28 in establishing and maintaining ESC identity? The observations reported by Melton and colleagues whereby the differentiation-promoting effects of let-7 are antagonized by expression of ESCC miRNAs may explain the very subtle effects on the differentiation status of ESCs upon Lin28 depletion. Despite an accumulation of mature let-7 miRNAs in these conditions, cell differentiation (as measured by the persistent expression of pluripotency markers) was largely unaffected (Melton et al., 2010; Viswanathan et al., 2008; Hagan et al., 2009; Heo et al., 2009). Interestingly, however, one of the first studies reporting the derivation of human of iPSCs utilized a slightly different cocktail of factors (Oct4, Sox2, Nanog, and Lin28) than had been used previously for the reprogramming of mouse fibroblast cells (Yu et al., 2007; Takahashi and Yamanaka, 2006). This observation, together with the recent report that antagonizing let-7 enhances cellular reprogramming, indicates that Lin28-mediated inhibition of let-7 expression promotes cell dedifferentiation during reprogramming (Melton et al., 2010). Indeed, it was found that Lin28 accelerates reprogramming through a predominately cell-division-rate-dependent mechanism (Hanna et al., 2009).

Perspectives

Recent studies have begun to uncover details of complicated miRNA gene regulatory networks that are essential for ESC self-renewal and proliferation (Figure 1). Further investigation of the mechanisms underlying self-renewal and pluripotency of

mammalian ESCs will be critical to understanding not only normal embryonic development but also somatic cell reprogramming and oncogenesis. Identification of additional stemness factors as well as genes and pathways regulated by miRNAs will be exciting areas of future pursuit. It will therefore be important to identify the mechanisms and factors controlling miRNA expression both at the transcriptional as well as the posttranscriptional level. Intriguingly, it was recently found that expression of a large cluster of (>50) miRNAs encoded within the imprinted Dlk1-Dio3 gene cluster on mouse chromosome 12 uniquely distinguishes most iPSCs from ESCs. This gene cluster is actively expressed in ESCs and pluripotent iPSCs but is silenced in iPSC clones with incomplete epigenetic remodeling and compromised developmental potential (Stadtfeld et al., 2010). It will be interesting to determine which, if any, of these many miRNAs may be critical for ESC and iPSC pluripotency and to identify the corresponding target genes.

Based on the notable similarities between the rapid cell division of undifferentiated ESCs and the inappropriately shortened cell cycle and abnormal cell growth associated with tumorigenesis, it is perhaps not surprising that numerous miRNA-based mechanisms are shared between stem cells and cancer. For example, ESCC miRNAs share a common seed sequence with a larger family of miRNAs (dubbed onco-miRs) known to promote cellular proliferation, including members of the miR-17-92 cluster and miR-106 (Esquela-Kerscher and Slack, 2006). These onco-miRs, like the ESCC miRNAs, may be acting by enhancing cell cycle progression and promoting cell dedifferentiation. Further evidence of the parallels between ESCs and cancer is provided by the expression of the pluripotency factors Lin28 and Lin28B in a subset of tumors. Indeed, oncogenic Lin28B and, less frequently, Lin28 are re-expressed in several cancer types, which is accompanied by reduced let-7 levels. This model is consistent with the fact that let-7 family members act as tumor suppressors by repressing known oncogenes including c-Myc, Hmga2, and Ras (Esquela-Kerscher and Slack, 2006). Thus, approaches involving manipulation of miRNA activity, i.e., small molecules to inhibit or promote miRNA function, may constitute alternative strategies to treat cancer, to improve the efficiency of cell reprogramming, and/or to drive ESC differentiation into particular cell types.

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